Journal of Gerontology: BIOLOGICAL SCIENCES
Cite journal as: J Gerontol A Biol Sci Med Sci. 2011 September;66A(9):965–971
doi:10.1093/gerona/glr103

Advance Access published on June 30, 2011

Aloe vera or Resveratrol Supplementation in Larval Diet Delays Adult Aging in the Fruit Fly, *Drosophila melanogaster*

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Longevity extension in *Drosophila melanogaster* by feeding diet supplemented with chemicals throughout adulthood can cause harmful side effects. We tested the effect of larval diet supplementation with five different concentrations of resveratrol and one concentration of *Aloe vera* extract on the adult longevity of short-lived *D melanogaster* populations. Resveratrol and *A vera* extract supplementation of larval diet extended adult longevity in both the male and female flies without reducing fecundity but by efficient reactive oxygen species scavenging through increased antioxidant enzymes activity and better neuroprotection as indicated by increased locomotor activity in adult males.

Key Words: Antioxidant enzymes—Neuroprotection—Fruit fly—Resveratrol—*Aloe vera*.

Received February 2, 2011; Accepted May 22, 2011

Decision Editor: Rafael de Cabo, PhD

LONGEVITY is an important fitness trait that is governed by both genetic and environmental components, and *Drosophila melanogaster* has many strengths as model organism to study longevity extension (1). Adult diet manipulation has been the principle means of longevity extension (1–4) as larval diet manipulation was ineffective (5,6). Longevity extension has also been achieved by feeding diet supplemented with drugs such as 4-phenylbutrate (7), morphine (8), and resveratrol (9,10) throughout adulthood. However, many studies in the recent past have questioned the efficacy of resveratrol in extending longevity (11–13). The disagreement between different studies could be due to (i) species-specific effects of resveratrol (13), (ii) differing diet composition in which resveratrol is mixed thus affecting availability for uptake, and/or (iii) differing adult physiology (6) due to different genetic background. Furthermore, feeding diet supplemented with chemicals throughout adulthood is akin to administering medication for achieving an intended target—in this case extension of longevity. It is a common knowledge that continuous medication can have adverse effects. Exploiting the strength of *D melanogaster* having distinct preadult and adult phase, we intended to test the age old adage "prevention is better than cure" by altering the larval diet and ascertaining the effects in adults, as selective pressures acting on preadult phase are known to alter the adult traits (14). We supplemented the larval diet with five different concentrations of resveratrol and one concentration of *Aloe vera* extract and assessed their effect on adult longevity and associated traits.

Supplementation of larval diet with resveratrol was able to extend the longevity of short-lived fruit fly populations in a dose-dependent manner. The longevity extension was not due to reduction in reproductive effort but was perhaps effected through multiple mechanisms involving efficient lipid utilization, prevention of neurodegeneration, regeneration of nerve fibers, and upregulation of antioxidant enzymes. Five milliliters of *A vera* extract supplementation mimics the effects of 50 μM resveratrol. Longevity extension by *A vera* extract is through multiple mechanisms other than calorie restriction.

Methods

Collection of Flies for Assays

The *D melanogaster* populations used in this study were derived from corresponding ancestral populations maintained on a 21-day egg-to-egg discrete generation cycle by transferring to single breeding cage, only the first 25% of the emerging flies from each of 120 vials, each with ~60 eggs in 6 mL standard media (SM). The vials were incubated at 25 ± 1°C, 75 ± 5% relative humidity, and 24:0 L:D cycle (hence forth referred to as SLC [Standard Laboratory Conditions]). Eggs for initiating the subsequent generation were collected on Day 3 postemergence. The populations had been selected for faster preadult development and early reproduction for nearly 430 generations before being used in this experiment. The populations were maintained for one generation without selection for faster preadult development prior to assaying the populations for various life-history

traits. Eggs were collected from the running cultures and dispensed into vials with about 6 mL of food at a density of ~50 eggs per vial. Forty such vials were set up per population. The vials were incubated at SLC, and a close vigil was maintained to ascertain the emergence of adults. All the flies that emerged by Day 8 from egg collection day were transferred into Plexiglass cages with abundant food supplemented with live yeast-acetic acid paste for 2 days (hence forth referred to as SF [Standardized Flies]). On Day 3, eggs were collected from SF by providing fresh uncontaminated nonnutritive agar plate. The eggs were dispensed into 6-mL media vials containing SM (standard control); 25, 50, 100, 200, or 800 μM resveratrol; 5 mL/L *A vera* extract; or 400 μL/L dimethyl sulfoxide (DMSO control) that was added to SM, just prior to pouring into vials. Forty vials with ~50 eggs per vial were set up for each treatment group per population. The vials with eggs were incubated at SLC. The emerging flies were collected into prelabeled holding vials with ~6 mL SM for 1 day, before being used in various assays.

Preparation of Standard and Aloe vera *Extract–Supplemented Media*

Banana (205 g), jaggery (35 g), yeast (36 g), and barley (25 g) ground to a fine paste in 180 mL water was added to agar (12.4 g) dissolved in 1,000 mL lukewarm water and brought to a boil on low flame. The media was cooled to 37°C, and benzoate (2.4 g) dissolved in ethanol (45 mL) was added and mixed thoroughly. This is referred to as SM. For treatment groups, 25, 50, 100, 200, or 800 μM resveratrol dissolved in DMSO; 5 mL *A vera* extract; or 400 μL DMSO was added to 1 L ready media postaddition of benzoate. For obtaining *A vera* (crude) extract, a single freshly cut healthy leaf was stored for about 24 hours in the freezer chamber of a refrigerator, de-skinned using a sterile blade, and clear liquid extracted by applying pressure (in a presterilized pestle and mortar) on the clear pulp. The *A vera* plants were maintained singly in pots containing sand, red soil, and farm yard manure in 2:6:2 ratio. The potted plants were watered once in 3 days.

Preadult Development Time and Viability Assay

Eggs were collected over a 1-hour window from SF and then dispensed into vials with 6 mL food at a density of exactly 50 eggs per vial. The vials were incubated at SLC. Ten such vials were set up per treatment group per population. Once the pupae darkened, the vials were checked regularly at 2-hour intervals and the number of eclosing males and females recorded. The adults were frozen immediately for use in the dry weight and lipid assay. The 2 hourly observations were continued until no flies eclosed for three consecutive days in any of the vials. The total number of flies that emerged from each of the vials was used to estimate the preadult viability in different treatment groups.

Adult Dry Weight and Lipid Assay

The freeze-killed flies from preadult development time assay were sorted under the binocular microscope. Females were distributed in groups of 10 into clean dry vials, dried at 70°C for 36 hours, and weighed to the nearest micrograms. Lipid content was estimated by defatting the flies following the method of Zwaan and colleagues (15) with a minor modification. After recording the dry weight, the flies were placed in 1.5-mL microcentrifuge tubes containing 1.3 mL ether. Lipid was extracted over a 36-hour period at room temperature of 25 ± 2°C with gentle agitation on a gel-rocker set to 20 rpm. Ether was changed every 12 hours. At the end of 36 hours, flies were removed from the ether, washed with 1 mL fresh ether, dried at 70°C for 2 hours, and weighed to obtain lipid-free dry weights. The difference between dry weight "before" and "after" ether extraction was taken as the total lipid content. Five vials were set up per treatment per population.

Fecundity Assay

Flies from the holding vials were sexed under light CO_2 anesthesia and single pair (one male + one female) were transferred to vial with ~3 mL SM. Twenty such vials were set up per treatment per population. Flies were transferred without anesthesia to fresh SM vials every 24 hours, and the eggs laid during the previous 24 hours were counted under binocular microscope and recorded. The daily egg counts were carried out till the death of the female fly in each test vial.

Longevity Assay

The longevity of reproducing flies was assayed. One-day-old flies were sorted into vials containing ~4 mL SM at a density of four mixed sex pairs (four females + four males) per vial. Twenty vials were set up per treatment per population. In all 640 female and 640 male flies were assayed for longevity. Mortality was recorded every 24 hours, the dead flies were removed from the vial, and the live flies were transferred to fresh SM vials every alternate day.

Climbing Assay

Climbing assay was carried out with male flies following the methods of Crowther and colleagues (16). Twenty flies were placed at the bottom of a 4″-long glass vial over which another 4″-long vial was inverted. Glass vials were separated from each other after 30 seconds, and the number of flies in the top vial was counted. Four climbing trials (akin to sampling with replacement) were conducted per vial. The climbing index was expressed as percentage of the number of flies that climbed to the top vial relative to the total number of flies tested ($n = 80$). Five replicate vials per treatment per population were assayed. Hence, a total of 400 sample flies were assayed per treatment per population.

Extract Preparation for Enzyme Activity

Flies from the holding vials were sexed under light CO_2 anesthesia and stored at −20°C before being used. Three replicates of 25 flies per sex per treatment per population were homogenized in ice-cold protein extraction buffer (20 mM Tris–HCl, pH 8.0; 1 mM EDTA; 1 mM phenyl methane sulfonyl floride; and 0.1% Triton-X 100), followed by centrifugation at 15,079*g* at 4°C for 30 minutes. The supernatant was used as crude enzyme extract for activity. Protein concentration was measured by using BCA-Protein Assay Kit (Sigma–Aldrich) according to manufacturers protocol.

Antioxidant Enzyme Assays

All spectrophotometric measurements were taken in a double-beam UV Visible spectrophotometer (UV-1800; Shimadzu, Japan).

Superoxide Dismutase Assay

Activity of SOD (EC 1.15.1.1) was determined by following the method of Beauchamp and Fridovich (17) with minor modifications. Activity buffer consisted of 100 mM KPO_4 buffer, pH 7.8; 0.05 μM EDTA; 45 mM l-methionine; 65 μM nitro-blue tetrazolium; and 35 μL of protein extract in a total volume of 3 mL. Riboflavin (2 mM) was added at the end and mixed. The tubes were kept under light source (20 W) for 30 minutes. Only assay activity buffer (including Riboflavin) was used as a control. Absorbance was measured at 560 nm. One unit of SOD activity (U) is defined as the amount of enzyme required to cause 50% inhibition in the rate of reduction of nitro-blue tetrazolium under specified conditions. Results were expressed as unit activity, units per milligram of protein.

Catalase Assay

Catalase activity (EC 1.11.1.6) was measured by using protocol of Aebi (18). The activity buffer consisted of 100 mM KPO_4 buffer, pH 7.0; 20 mM H_2O_2; and 50 μL enzyme extract in a total volume of 1 mL. Phosphate buffer and enzyme extracts were added to a cuvette, and the reaction was initiated by adding H_2O_2. Decrease in H_2O_2 was monitored by measuring absorbance at 240 nm continuously for 2 minutes with intervals of 30 seconds using the auto-rate assay mode on spectrophotometer. One unit of enzyme is the amount of enzyme required to convert 1 mol of H_2O_2 to product in 1 second. Enzyme activity was expressed as units per milligram of protein.

Statistical Analyses

In all cases except survival function analysis, the population means were used as the units of analysis. The significance of the difference between means was assessed using one-way analysis of variance. The difference among treatments were compared by Tukey–Kramer Minimum Significant Difference ($MSD\alpha_{0.05}$) Test (19). The significance of the difference between adult survival curves was analyzed using Kaplan–Meier log-rank test (20).

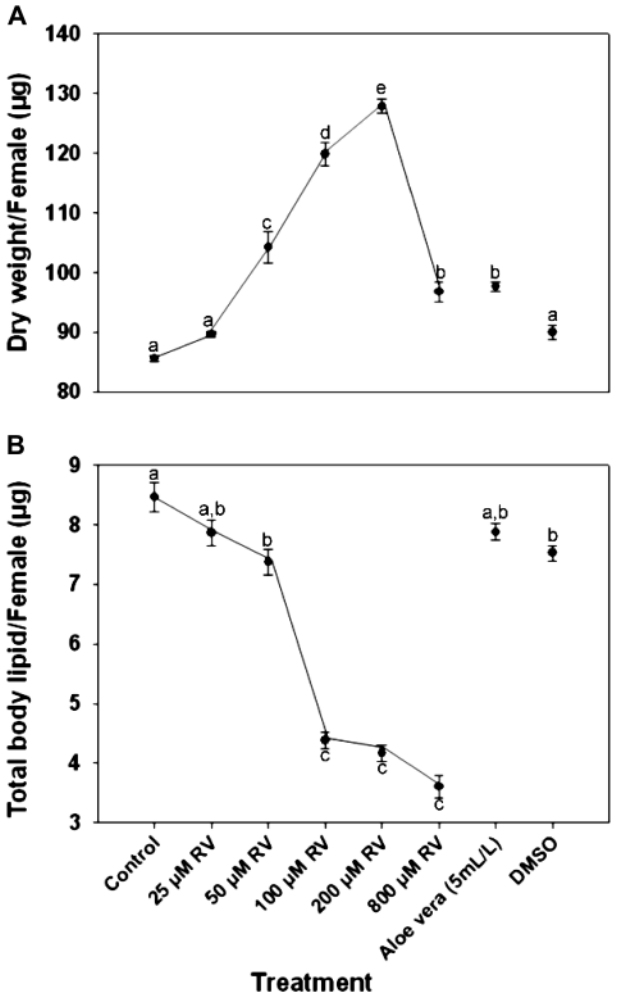

Figure 1. Mean (±SE) dry weight (**A**) and total body lipids (**B**) per female fly. Data points having same alphabets are not significantly different from each other, whereas those having different alphabets are significantly different from each other (Tukey–Kramer $MSD\alpha_{0.05}$: dry weight = 7.1761 μg, total body lipids = 0.8916 μg).

Results

Adult Dry Weight and Total Body Lipid Content

There was a significant effect of diet supplementation with resveratrol and *A vera* on the dry weight ($F_{7,16}$ = 106.7825, p = .000) and lipid content ($F_{7,16}$ = 119.0759, p = .000) of the female flies. The dry weight increased almost exponentially with increasing dose of resveratrol up to 200 μM. The lowest dose was ineffective in increasing the weight compared with standard control and DMSO control. The effect of 800 μM resveratrol was similar to 5 mL *A vera* extract, both of which were statistically significantly higher than standard control, DMSO control, and 25 μM of resveratrol supplementation but lower than 50, 100, and 200 μM resveratrol supplementation (Figure 1A). The lipid content decreased

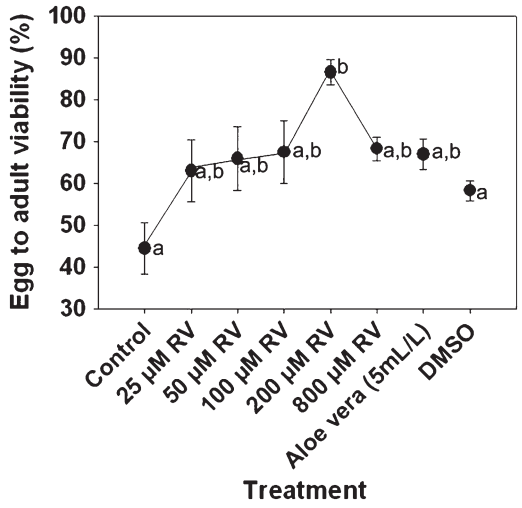

Figure 2. Mean (±SE) egg to adult viability. Data points having same alphabets are not significantly different from each other, whereas those having different alphabets are significantly different from each other (Tukey–Kramer $MSD\alpha_{0.05} = 26.7633$).

with increasing resveratrol dose. The effect of *A vera* supplementation was not different from the standard control and DMSO control (Figure 1B).

Preadult Development Time and Viability

Diet supplementation with resveratrol and *A vera* had no significant effect on the preadult development time of both male ($F_{7,16} = 0.3251$, $p = .931$) and female ($F_{7,16} = 0.4820$, $p = .834$) flies. However, the preadult viability significantly increased with supplementation of resveratrol and *A vera* ($F_{7,16} = 4.5781$, $p = .006$). Post hoc comparisons showed that the standard control and DMSO control were not significantly different from each other as well as 25, 50, 100, and 800 µM resveratrol or 5 mL *A vera* treatment groups but were significantly different from 200 µM resveratrol treatment group. Furthermore, there was no significant difference in the preadult viability of flies from different concentrations of resveratrol and *A vera* extract (Figure 2). The unidirectional change in the viability of all diet supplementation treatments from controls must have resulted in overall significance as indicated by analysis of variance.

Longevity and Fecundity

Supplementation of diet with resveratrol and *A vera* significantly changed the average longevity of female flies ($F_{7,16} = 5.4159$, $p = .005$) but not of male flies ($F_{7,16} = 2.984$, $p = .10$). The median life span of both female and male flies was not significantly altered by diet supplementation. However, the maximum life span was significantly altered in both the sexes (Table 1). The increase in the longevity of females due to resveratrol supplementation was nonlinearly dose dependent. The increase in longevity of female flies was not linked to loss of fertility as there was no significant effect of diet supplementation with resveratrol and *A vera* on lifetime fecundity ($F_{7,16} = 2.0296$, $p = .114$; Figure 3). Two hundred micro molars of resveratrol supplementation had the maximum effect in delaying death. The effect of *A vera* was similar to 50 µM resveratrol. Average, median, and maximum longevity are descriptive statistics. Average can be greatly influenced by extreme values, and median and maximum values are one datum each in the entire data set. Descriptive statistics provide overall comparisons and do not compare the progression of a biological process—in this case, survival probabilities. Hence, we compared the survival functions of flies raised on different concentrations of resveratrol and one concentration of *A vera*. The survival

Table 1. Mean, Median, and Maximum Longevity (days) of Adult *Drosophila melanogaster* Flies, Reared as Larvae on Diet Supplemented with Five Different Concentrations of Resveratrol or One Concentration of *Aloe vera* Extract*

		Female									Male								
		Mean			Median			Maximum			Mean			Median			Maximum		
Treatment	*N*	R1	R2	R3	R1	R2	R3	R1	R2	R3	R1	R2	R3	R1	R2	R3	R1	R2	R3
Control	80	15.696	18.863	20.532	15	20	19	33	32	38	13.75	22.20	19.26	14	24	18	22	35	36
25 µM	80	16.088	20.738	24.646	15	21	25	25	35	44	13.81	23.46	20.28	14	26.5	21	24	35	41
50 µM	80	20.418	24.863	27.671	19	25	29	39	40	53	19.46	26.75	25.73	17	28.5	26	42	42	49
100 µM	80	25.987	30.949	29.910	26	30	31	42	58	55	23.00	26.40	27.63	21	27	28.5	40	54	54
200 µM	80	27.346	32.344	39.392	28	30	33	42	58	59	23.13	27.21	29.42	22.5	28	30	45	64	57
800 µM	80	19.450	22.550	24.671	20.5	23	25	34	37	39	20.41	20.08	22.58	21	20.5	23	42	39	44
A vera	80	23.050	21.913	27.266	23	23	27	41	36	48	24.36	27.64	27.00	27	29	28	37	45	49
DMSO	80	21.538	23.063	23.899	22	25	24	39	37	41	19.29	23.64	22.33	20	26	22	35	36	38
F			5.4159			0.5648			3.3987			2.9841			1.7251			4.5155	
p			<.005			NS			<.05			>.10			>.50			<.02	
Tukey–Kramer $MSD\alpha_{0.05}$			9.871†						19.271†									18.843†	

Notes: DMSO = dimethyl sulfoxide; NS = not significant.

*Each of the three replicate populations (R1, R2, and R3) had 20 mixed sex (four female + four male) vials, thus giving a total sample size of 80 flies/replication/sex/treatment.

†Tukey–Kramer $MSD\alpha_{0.05}$ values for comparison of difference among treatments are provided.

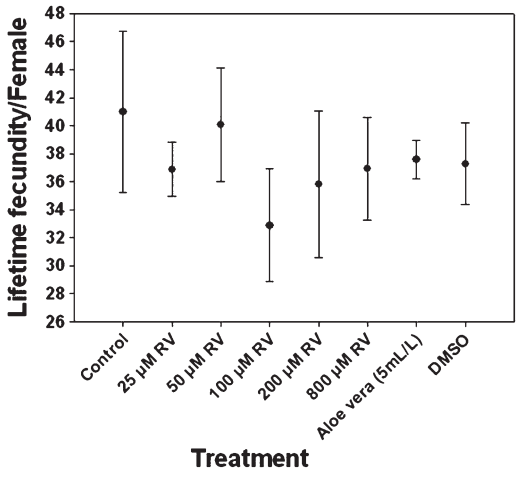

Figure 3. Mean (±SE) lifetime fecundity was not significantly different between treatment groups ($F_{7,16}$ = 2.0296, p = .114).

rates of both female and male flies were significantly affected by resveratrol and *A vera* supplementation (Figure 4).

Locomotor Activity

Male locomotor activity was significantly influenced by diet supplementation with resveratrol and *A vera* ($F_{7,16}$ = 138.7598, p = .000). Flies reared on 200 μM resveratrol were the most active, whereas the standard control flies were the least active (Figure 5).

Superoxide Dismutase Activity

Superoxide dismutase activity was significantly upregulated in male ($F_{7,16}$ = 66.4002, p = .000) and female ($F_{7,16}$ = 18.9459, p = .000) flies by resveratrol and *A vera* supplementation (Figure 6A).

Catalase Activity

Both resveratrol and *A vera* supplementation had a significant effect on the catalase activity in male ($F_{7,16}$ = 84.0003, p = .000) and female ($F_{7,16}$ = 24.5205, p = .000) flies (Figure 6B).

Discussion

In the present article, we tested the effects of resveratrol and *A vera* extract on key life-history traits of *D melanogaster* populations selected for faster preadult development and early reproduction for 430 generations. The populations had evolved significantly smaller size, lower lipid reserves, shorter life span, and reduced fecundity as correlated responses (14,21,22). Unlike in the earlier studies (3,14,15), the significant increase in the dry weight was not accompanied by increase in development time. Despite having reduced lipid content, resveratrol and *A vera* supplementation increased longevity without reducing fecundity, unlike diet manipulation studies that reported increase in life span by compromising reproduction (2,3,5). Our results suggest that there is no causal link between lipid reserves, longevity, and reproductive effort. Similar results were reported in the nematode, *Caenorhabditis elegans*; the fruit fly, *D. melanogaster* (9); and the fish, *Nothobranchius furzeri* (23). Longevity extension by resveratrol and *A vera* is associated with the lowering of the time-dependent death rate (Figure 4). A similar effect is reported for *N furzeri* (23). The increased longevity of flies reared as larvae on resveratrol-supplemented diet

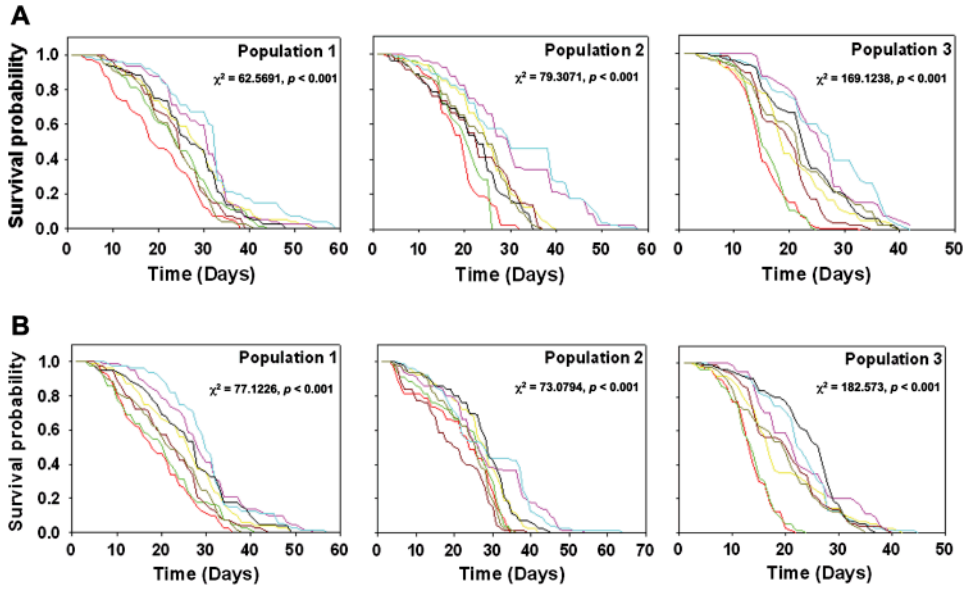

Figure 4. Kaplan–Meier survival curves for standard control (red), dimethyl sulfoxide control (olive green), 25 μM resveratrol (light green), 50 μM resveratrol (yellow), 100 μM resveratrol (megenta), 200 μM resveratrol (light blue), 800 μM resveratrol (brown), and 5 mL *Aloe vera* extract (black). Comparison of age-dependent survival curves of female (**A**) and male (**B**) flies of three different populations indicated highly significant difference.

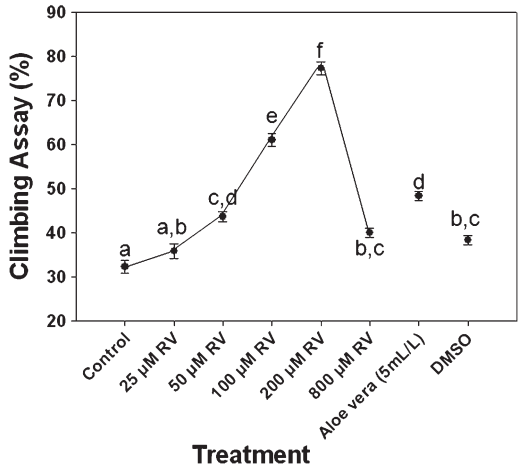

Figure 5. Locomotor activity of male flies. Data points having same alphabets are not significantly different from each other, whereas those having different alphabets are significantly different from each other (Tukey–Kramer $MSD\alpha_{0.05} = 6.2686$).

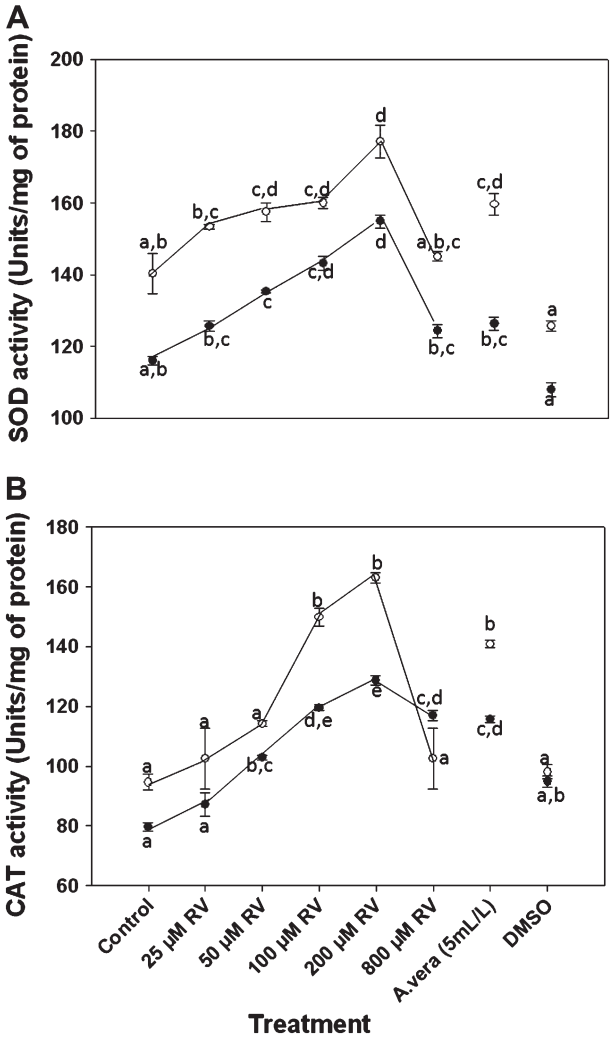

Figure 6. Antioxidant enzyme activity in male (solid circles) and female (open circles) flies. (**A**) SOD. (**B**) Catalase. Data points having same alphabets (within sex comparisons) are not significantly different from each other, whereas those having different alphabets are significantly different from each other (Tukey–Kramer $MSD\alpha_{0.05}$: $SOD_{male} = 9.0689$, $SOD_{female} = 18.1643$, $catalase_{male} = 9.2327$, $catalase_{female} = 26.3186$).

were associated with significantly higher body mass but lower body lipids at emergence compared with the standard controls and DMSO controls, suggesting that longevity extension by resveratrol could have been due to efficient lipid utilization resulting in the production of fewer reactive oxygen species and/or better scavenging of reactive oxygen species exerted by upregulating detoxifying enzymes—SOD and catalase. Our results and views are in agreement with the resveratrol-dependent upregulation of detoxification enzymes in the brain of healthy rats (24). The increased locomotor activity of flies reared on media supplemented with resveratrol could have been due to its neuroprotective activity (24–29). In another study, feeding fish with resveratrol-supplemented diet prevented age-dependent neurodegeneration (23). In addition to neuroprotection, resveratrol could have stimulated the growth and regeneration of nerve fibers that could have resulted in increased preadult viability, because the larval growing media was modified in this study, unlike all other studies that altered the adult diet.

The earlier studies that attempted to understand the effect of resveratrol in modulating longevity of invertebrates as well as vertebrates fed the adults with the modified diet on a regular basis, suggesting that continuous supply of resveratrol is essential for maintaining the system in a healthy condition. Our study suggests that one time supplementation of the larval diet with resveratrol is enough to mimic the continuous diet supplementation of adult flies.

The lipid content of the flies raised as larvae on *A vera* extract–supplemented diet was not different from that of flies from SM, suggesting that *A vera* extends longevity through mechanisms other than calorie restriction, as increased longevity in dietary restriction studies were associated with increased lipid content and reduced dry weight and fecundity (30). Longevity extension by *A vera* is probably mediated through prevention of neurodegeneration and/or regeneration of nerve fibers as indicated by increased locomotor activity. Increased activity of detoxifying enzymes SOD and catalase seems to be able to clear the system of the toxic elements that could have otherwise caused damage and lead to no improvement in longevity. *Aloe vera* is known to contain a plethora of phytochemicals, such as 1,8-dihydroxyanthraquinone derivatives and their glycosides, proteins, lipids, amino acids, vitamins, enzymes, inorganic compounds, and small organic compounds (31), some, if not most of them could have contributed to improved health through correction of many metabolic processes—anthraquinone is a starting material for production of antioxidants to cite one example.

Aloe vera extract seems to mimic the longevity extension effects of resveratrol as well as morphine (8) through regeneration of nerve fibers, neuroprotection as indicated by increased locomotor activity, and upregulation of detoxifying enzymes.

Funding

This work was supported in part by funds from University of Delhi and Council of Scientific and Industrial Research, Government of India to M.S.

Acknowledgments

We thank two anonymous reviewers whose comments helped us in improving the manuscript and Prof. Amitabh Joshi (Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore) for providing fly populations used in this work. K.T.C. thanks Department of Biotechnology, Government of India for the postdoctoral fellowship.

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